APPENDIX A

CROSS-LINKING

cross-linking

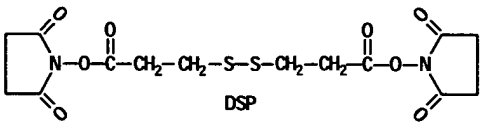
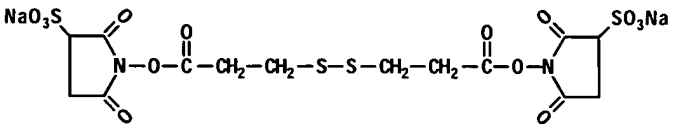
Applications for Use of Cross-linkers

Cell Surface Cross-linking

To ensure cell-surface specific cross-linking for identification of surface receptors or their ligands, it is best to use membrane-impermeable cross-linkers. In the past, researchers used water-insoluble cross-linkers and carefully controlled the amount of cross-linker and the cross-linking duration. This prevented penetration of the membrane by the cross-linker and subsequent reaction with membrane proteins. Many references cite the use of membrane-permeable cross-linkers for cell surface cross-linking. Staros developed water-soluble sulfo-NHS analogs as alternatives to membrane permeable, homobifunctional NHS-ester and imidoester cross-linkers.[26] The sulfo-NHS-ester, homobifunctional cross-linker BS_3 (Product #21579) is very useful for cell surface cross-linking of ligands to receptors through primary amines on each. The sulfonyl groups attached to the succinimidyl rings of sulfo-NHS cross-linkers make them membrane-impermeable and non-reactive with inner membrane proteins. Therefore, cross-linking time and quantity of cross-linker are less critical when using sulfo-NHS-esters. Pierce offers a variety of sulfo-NHS-ester cross-linkers, both homobifunctional and heterobifunctional. Homobifunctional sulfo-NHS-esters, heterobifunctional sulfo-NHS-esters and photoreactive phenyl azides are good choices for cross-linking on the surface of a cell. See Tables 3, 5 and 9 for specific characteristics and selection of cross-linkers for cell surface applications.

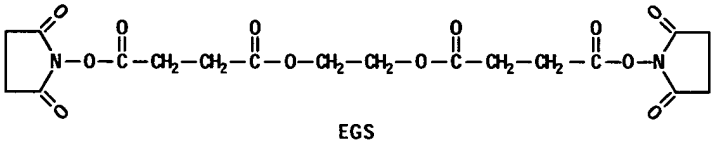
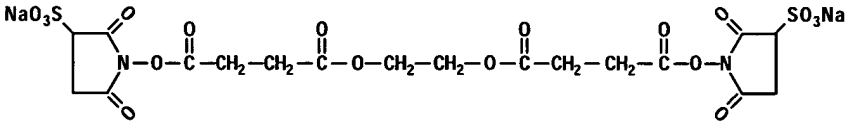
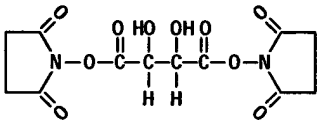
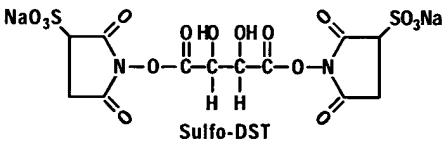
cross-linking

Table 3: Homobifunctional NHS-Ester Cross-linkers (Continued)

CROSS-LINKER	PRODUCT #	M.W.	SPACER ARM LENGTH	REACTIVITY/ CHARACTERISTICS	APPLICATIONS/ REFERENCES
DSP Dithiobis(succinimidyl propionate)  DSP	22585	404.42	12 Å	One of the most widely used cross-linkers, also known as Lomant's Reagent.[41] Water-insoluble, thiol-cleavable—can be cleaved with 10-50 mM DTT at 37°C for 30 minutes or with 5% β-mercaptoethanol in SDS-PAGE sample buffer (2% SDS, 6.25 mM Tris base, 10% glycerol) at 100°C for 5 minutes.	• Examining spatial relationships of the capsid polypeptides of the mengo virion[91] • Studying renal Na+ and K+-ATPase[92] • Nearest neighbor relationships of bovine mitochondrial H+-ATP[93] • Producing interactions between protein components of the chemotaxis mechanism in E. coli[94] • Chemical cross-linking of a-CPI[95] • Identifying cross-linked cytochrome P-450 in rat liver microsomes[96] • Studying the influence of metal ions on prothrombin self-association[97] • Studying glycoprotein topology on intact human red blood cells[98] • Molecular identification of receptors for vasoactive intestinal peptide in rat intestinal epithelium[99] • Characterization of a cell surface receptor for colony-stim ulating factor (CSF-2a)[100] • Determining membrane antigens by covalent cross-linking to monoclonal antibodies[101]
DTSSP [3,3'-Dithiobis(sulfosuccinimidyl propionate)  DTSSP	21577	608.51	12 Å	Water-soluble analog of DSP[86]	• Cross-linking the extracytoplasmic domain of the anion exchange channel in intact human erythrocytes[88] • Cross-linking studies on Novikoff ascites hepatoma cytokeratin filaments[89] • Characterization of the B lymphocyte Fc receptor for IgE[90] • Cross-linking platelet glycoprotein Ib[102] • Characterization of a membrane-ribosome complex in B. subtilis[103]

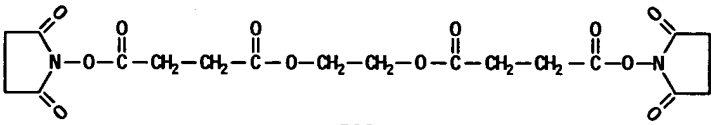
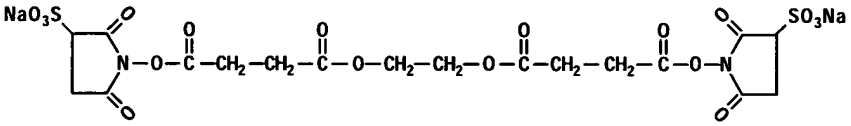
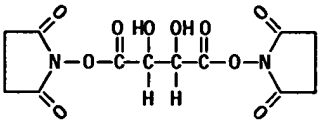
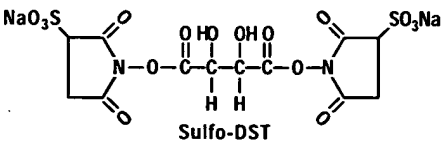
cross-linking

Table 3: Homobifunctional NHS-Ester Cross-linkers (Continued)

CROSS-LINKER	PRODUCT #	M.W.	SPACER ARM LENGTH	REACTIVITY/ CHARACTERISTICS	APPLICATIONS/ REFERENCES
EGS Ethylene glycobis (succinimidylsuccinate)  EGS	21565	456.37	16.1 Å	Water-insoluble hydroxylamine. Cleavable—cleaved by incubating with 1 M hydroxylamine for 3-6 hours at 37°C at pH 8.5. Lactose dehydrogenase retained 60% of its activity after cross-linking with EGS.[104]	• Cross-linking studies of cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase[105] • Tumor necrosis factor (TNF) and lymphotoxin LT cross-linking[106] • Converting a gonadotropin-releasing hormone antagonist to an agonist[107] • Preparing the EGS dimer of the GnRH agonist D-Lys-GnRH[108] • Covalent cross-linking of vasoactive peptide to its receptors on intact human lymphoblasts[76] • Binding and cross-linking of ^{125}I-gastrin releasing peptid (GRP)[109]
Sulfo-EGS Ethylene glycobis(sulfo-succinimidylsuccinate)  Sulfo-EGS	21566	660.47	16.1 Å	Water-soluble analog of EGS. Reactions similar to EGS.[104]	104
DST Disuccinimidyl tartarate  DST	20590	344.24	6.4 Å	Water-insoluble sample cross-linked with DST in first-dimensional gel. Cleavable by soaking in 0.015 M sodium periodate, 0.1% SDS, 0.02 M sodium phosphate, pH 7.0 for 2 hours (with several changes) at room temperature.[110]	• Cross-linking of ubiquinone cytochrome c reductase (complex III)[110] • Characterization of the cell surface receptor for colony-stimulating factor (CSF-2a)[111] • Cross-linking study of the Ca^{2+}, Mg^{2+} activated adenosine triphosphate of E. coli[100] • Human promyelocytic cell line cross-linking of cell lysate with DST[112]
Sulfo-DST Disulfosuccinimidyl tartarate  Sulfo-DST	20591	548.34	6.4 Å	Water-soluble analog of DST	100,110-112

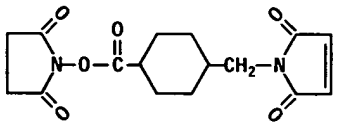
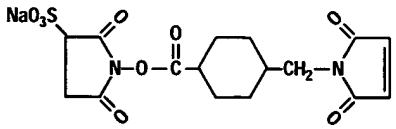
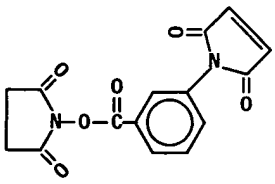
cross-linking

Table 3: Homobifunctional NHS-Ester Cross-linkers (Continued)

CROSS-LINKER	PRODUCT #	M.W.	SPACER ARM LENGTH	REACTIVITY/ CHARACTERISTICS	APPLICATIONS/ REFERENCES
EGS Ethylene glycobis (succinimidylsuccinate)  EGS	21565	456.37	16.1 Å	Water-insoluble hydroxylamine. Cleavable—cleaved by incubating with 1 M hydroxylamine for 3-6 hours at 37°C at pH 8.5. Lactose dehydrogenase retained 60% of its activity after cross-linking with EGS.[104]	• Cross-linking studies of cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase[105] • Tumor necrosis factor (TNF) and lymphotoxin LT cross-linking[106] • Converting a gonadotropin-releasing hormone antagonist to an agonist[107] • Preparing the EGS dimer of the GnRH agonist D-Lys-GnRH[108] • Covalent cross-linking of vasoactive peptide to its receptors on intact human lymphoblasts[76] • Binding and cross-linking of ^{125}I-gastrin releasing peptid (GRP)[109]
Sulfo-EGS Ethylene glycobis(sulfo-succinimidylsuccinate)  Sulfo-EGS	21566	660.47	16.1 Å	Water-soluble analog of EGS. Reactions similar to EGS.[104]	104
DST Disuccinimidyl tartarate  DST	20590	344.24	6.4 Å	Water-insoluble sample cross-linked with DST in first-dimensional gel. Cleavable by soaking in 0.015 M sodium periodate, 0.1% SDS, 0.02 M sodium phosphate,. pH 7.0 for 2 hours (with several changes) at room temperature.[110]	• Cross-linking of ubiquinone cytochrome c reductase (complex III)[110] • Characterization of the cell surface receptor for colony-stimulating factor (CSF-2a)[111] • Cross-linking study of the Ca^{2+}, Mg^{2+} activated adenosine triphosphate of E. coli[100] • Human promyelocytic cell line cross-linking of cell lysate with DST[112]
Sulfo-DST Disulfosuccinimidyl tartarate  Sulfo-DST	20591	548.34	6.4 Å	Water-soluble analog of DST	100,110-112

cross-linking

Table 5: NHS-Ester-Maleimide Heterobifunctional Cross-linkers

CROSS-LINKER	PRODUCT #	M.W.	SPACER ARM LENGTH	REACTIVITY/ CHARACTERISTICS	APPLICATIONS/ REFERENCES
SMCC Succinimidyl 4-(N-maleimido-methyl) cyclo-hexane-1-carboxylate  SMCC	22320	334.33	11.6 Å	Water-insoluble, noncleavable, very stable. Maleimide-reactive group.	• Conjugation of glucose oxidase from Aspergillus niger to rabbit antibodies[46] • Conjugating Fab' to horseradish peroxidase[116-119, 125] • Conjugating affinity-purified antidigoxin $F(ab')_2$ fragments to ß-galactosidase[120] • Enzyme labeling of antibodies and antibody fragments[121] • Conjugating alkaline phosphatase and human IgG $F(ab')_2$ fragments for phase change immunoassays[122] • Preparing immunogens [123,124]
Sulfo-SMCC Sulfo-succinimidyl 4-(N-maleimidomethyl) cyclo-hexane-1-carboxylate  Sulfo-SMCC	22322	436.37	11.6 Å	Water-soluble analog of SMCC; very stable maleimide-reactive group. This noncleavable cross-linker is membrane impermeable.	• A comparison of maleimide containing heterobifunctional cross-linkers in the conjugation of Fab' fragments to horseradish peroxidase[126] • Preparation of enzyme-antibody conjugates[127]
MBS m-Maleimidobenzoyl-N-hydroxysuccinimide ester  MBS	22310	314.2	9.9 Å	Water-insoluble, noncleavable cross-linker	• Preparing an insulin-ß-galactosidase conjugate[128] • Conjugating hen egg ovalbumin with thiolated synthetic copolymers of D-glutamic acid and D-lysine[129] • Preparing antibody-ß-galactosidase conjugates[130] • Producing ricin immunotoxins[131, 142] • Preparing hapten-carrier protein conjugates from peptides[132, 137, 138, 140, 141] • Coupling blasticidin S to bovine serum albumin[133] • Preparing Fab'-ß-galactosidase conjugates[134] • Preparing synthetic peptide antigens for making antibodies to detect oncogene-related proteins[135] • Investigating the mechanism of cytotoxicity of diphtheria toxin coupled to anti-CD3 MAb[136] • Preparing enzyme labeled viomycin[139]

cross-linking

Table 5: NHS-Ester-Maleimide Heterobifunctional Cross-linkers (Continued)

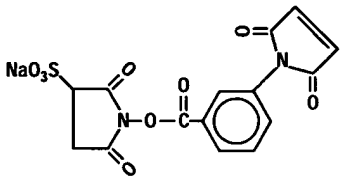
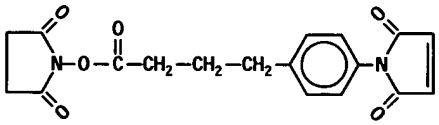
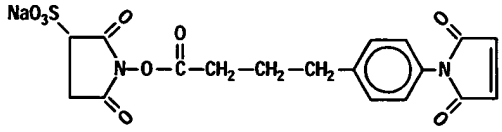
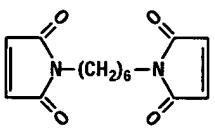
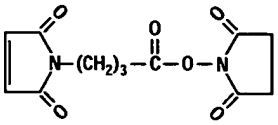
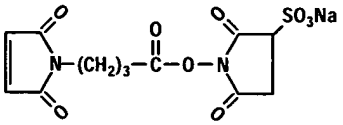
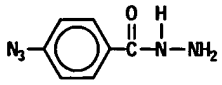
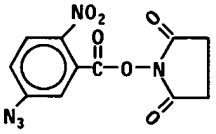
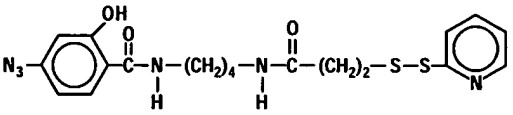
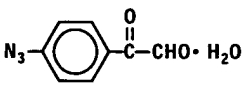
CROSS-LINKER	PRODUCT #	M.W.	SPACER ARM LENGTH	REACTIVITY/ CHARACTERISTICS	APPLICATIONS/ REFERENCES
Sulfo-MBS m-Maleimidobenzoyl-N-hydroxysulfosuccinimide ester  Sulfo-MBS	22312	416.24	9.9 Å	Water-soluble analog of MBS; noncleavable, membrane impermeable.	• An alternative method utilizing small quantities of ligand for affinity purification of monospecific antibodies[143] • Coupling of antibody to ß-D-galactosidase[144]
SMPB Succinimidyl 4-(p-maleimido-phenyl)-butyrate  SMPB	22315	356.32	14.5Å	Water-insoluble, extended spacer arm to limit steric hindrance; noncleavable.	• Conjugation of preformed vesicles and Fab' fragments in a study of liposomes as a carrier system[144] • Attaching insulin molecules to reconstituted Sendai virus envelopes[145] • Targeting of loaded Sendai virus envelopes by covalently attached insulin molecules to virus receptor-depleted cells[146] • Forming alkaline phosphatase-Fab' fragment conjugates for an enzyme immunoassay system[147] • Preparing peptide-protein immunogens[148]
Sulfo-SMPB Sulfosuccinimidyl 4-(p-maleimidophenyl)-butyrate  Sulfo-SMPB	22319	458.36	14.5Å	Water-soluble analog of SMPB. Extended spacer arm to limit steric hindrance; non-cleavable, membrane impermeable.	• Studying the transport of the varient surface glycoprotein of Trypanosome brucia[149] • Using aromatic cross-linkers such as Sulfo-SMPB to improve the yield of immunotoxin conjugates[142]
BMH Bismaleimidohexane  BMH	22319	276.29	16.1Å	Water-insoluble homobifunctional cross-linker employing two maleimide functional groups; noncleavable.	• Structural and functional studies of cross-linked Go_{150} protein subunits[150] • Studies of lymphocyte function-associated antigen-3 (LFA-3)[151] • Producing multimeric forms of CD4[152]

Table 5: NHS-Ester-Maleimide Heterobifunctional Cross-linkers (Continued)

CROSS-LINKER	PRODUCT #	M.W.	SPACER ARM LENGTH	REACTIVITY/ CHARACTERISTICS	APPLICATIONS/ REFERENCES
GMBS N-(g-maleimidobutyryloxy) succinimide ester	22314	280.24	10.2 Å	Water-insoluble, noncleavable.	• Acylation of antibody to introduce maleimide groups[153]
 GMBS					
Sulfo-GMBS N-(g-maleimidobutyryloxy) sulfosuccinimide ester	22324	382.28	10.2 Å	Water-soluble analog of GMBS; noncleavable, membrane impermeable.	154-155
 Sulfo-GMBS					

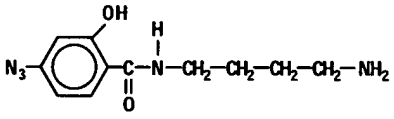
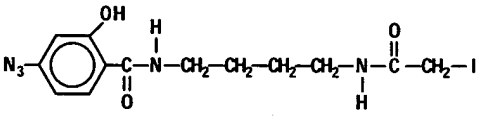
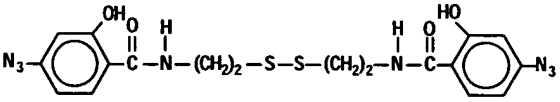
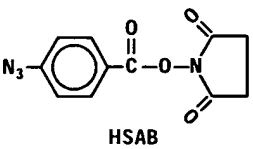
cross-linking

Table 9: Photoreactive Cross-linkers

CROSS-LINKER	PRODUCT #	M.W.	SPACER ARM LENGTH	REACTIVITY	APPLICATIONS/ REFERENCES
ABH Azidobenzoyl Hydrazide  ABH	21510 21509	177.17		• Hydrazide • Phenylazide	• Glycoprotein receptor studies[168]
ANB-NOS N-5-Azido-2-nitrobenzoyloxysuccinimide  ANB-NOS	21551	305.21	7.7 Å	• NHS-ester • Phenylazide	• Cross-linking cobra venom phospholipase A2 aggregation state[169] • Photo-cross-linking of the signal sequence of nascent preprolactin to a polypeptide of the signal recognition particle[170]
APDP N-[4-(p-azidosalicylamido) butyl]-3'(2'-pyridyldithio) propionamide  APDP	27720	446.55		• Pyridyl disulfide • Phenylazide	• Cross-linking of protein subunits and ligand by introduction of disulfide bonds[171]
APG *p*-Azidophenyl glyoxal monohydrate  APG	20107	193.16	9.3 Å	• Phenylazide • Phenyl glyoxal	• Inhibiting bovine heart lactic dehydrogenase, eggwhite lysozyme, horse liver alcohol dehydrogenase, and yeast alcohol dehydro genase[172] • Cross-linking ribonucleic acid-protein in E. coli ribosomes[173] • Identifying regions of brome mosaic virus coat protein chemically cross-linked in situ to viral RNA[174]

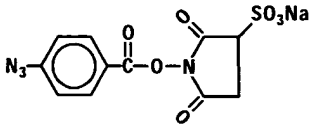
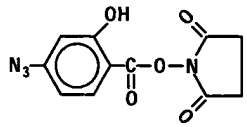
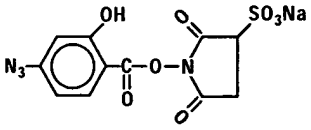
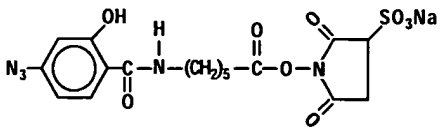
cross-linking

Table 9: Photoreactive Cross-linkers (Continued)

CROSS-LINKER	PRODUCT #	M.W.	SPACER ARM LENGTH	REACTIVITY	APPLICATIONS/ REFERENCES
ASBA 4-(*p*-Azidosalicyl-amido)butylamine  ASBA	21512	249.27	16.3 Å	• Carbonyl reactive • Phenylazide	
ASIB 1-(*p*-Azidosalicyl-amido)-4-(iodo-acetamido)butane  ASIB	21511	417.21	18.8 Å	• Iodoacetyl • Phenylazide	
BASED Bis-[β-4-azidosalicylamido)ethyl] disulfide  BASED	21564	474.54		• Phenylazide (homobifunctional)	• Receptor location • Near neighbor analysis • Protein structural studies • Appropriate in the absence of primary amines and thiols
HSAB N-Hydroxysuccinimidyl-4-azidobenzoate  HSAB	21560	260.21	8.0 Å	• NHS-ester • Phenylazide	• Photoaffinity labeling of peptide hormone binding sites[175] • Photoaffinity labeling of insulin receptor with an insulin analog[176] • Identifying nerve growth factor receptor proteins in sympathetic ganglia membranes[177] • Photoaffinity labeling the hormone receptor of both α and β subunits of human choriogonadotropin[178] • Isolating *in situ* cross-linked ligand-receptor complexes[179] • Cross-linking vasoactive intestinal polypeptide to its receptors on intact human lymphocytes[76]

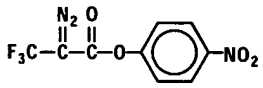
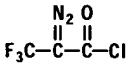
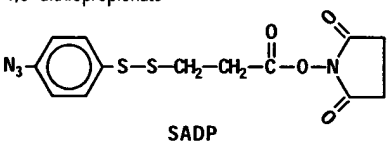
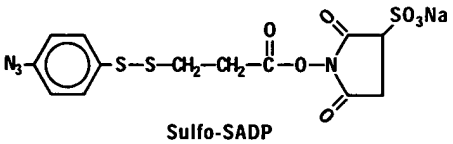
cross-linking

Table 9: Photoreactive Cross-linkers (Continued)

CROSS-LINKER	PRODUCT #	M.W.	SPACER ARM LENGTH	REACTIVITY	APPLICATIONS/ REFERENCES
Sulfo-HSAB N-Hydroxysulfo-succinimidyl 4-azidobenzoate	21561	362.25	9.0 Å	• NHS-ester • Phenylazide	• Photoaffinity labeling of peptide hormone binding sites[175] • See applications for HSAB
 **Sulfo-HSAB**					
NHS-ASA N-Hydroxysuccinimidyl-4-azidosalicylic acid	27715	276.21	8.0 Å	• NHS-ester • Phenylazide	• Photoaffinity labeling of ^{125}I-AS-Con A to erythrocyte ghosts[180] • Derivatizing human choriogonadotropin with ^{125}I-NHS-ASA and photo-cross-linking the αβ dimer[181] • Radiolabeling D-glucose and cross-linking the sugar to the human erythrocyte mono saccharide transporter[182] • Photoaffinity labeling of a bacterial sialidase[183]
 **NHS-ASA**					
Sulfo-NHS-ASA N-Hydroxysulfo-succinimidyl-4-azidosalicylic acid	27725	378.25	8.0 Å	• NHS-ester • Phenylazide	• See applications/references for NHS-ASA
 **Sulfo-NHS-ASA**					
Sulfo-NHS-LC-ASA Sulfosuccinimidyl-(4-azidosalicylamido)-hexanoate	27735	491.41	18 Å	• NHS-ester • Phenylazide	• See applications/references for NHS-ASA
 **Sulfo-NHS-LC-ASA** **M.W. 491.41** **Spacer Arm 18Å**					

cross-linking

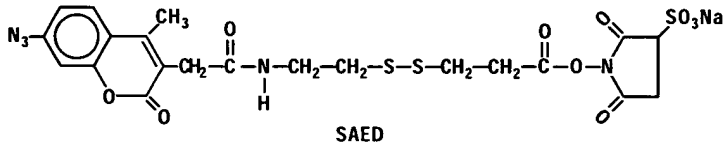
Table 9: Photoreactive Cross-linkers (Continued)

CROSS-LINKER	PRODUCT #	M.W.	SPACER ARM LENGTH	REACTIVITY	APPLICATIONS/ REFERENCES
PNP-DTP *p*-Nitrophenyl- 2-diazo-3,3,3-trifluoropropionate  **PNP-DTP**	20669	276.15		• Diazo	• Photoaffinity labeling of thyroid hormone nuclear receptors in intact cells[184,185]
DTP 2-Diazo-3,3,3,-trifluoro- propionylchloride  **DTP**	20670			• Sulfhydryls • Amines	• Pierce offers this product for researchers who require the acid chloride precursor of PNP-DTP
SADP N-succinimidyl- (4-azidophenyl) 1,3'-dithiopropionate  **SADP**	21552	352.38	13.9 Å	• NHS-ester • Phenylazide	• Cross-linking concanavalin A to receptors on the human erythrocyte membrane[186] • Preparing photoactivatable glycopeptide reagents for site-specific labeling of lectins[187] • Attaching a Sendai virion envelope and a mouse surface membrane polypeptide on newly infected cells[188] • Cross-linking platelet glycoprotein 1b[102]
Sulfo-SADP Sulfosuccin (4-azidophenyldithio) propionate  **Sulfo-SADP**	21553	454.45	13.9 Å	• NHS-ester • Phenylazide	• See applications/references for SADPimidyl-

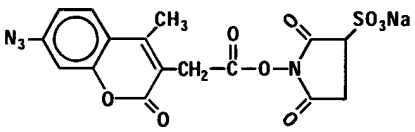
cross-linking

Table 9: Photoreactive Cross-linkers (Continued)

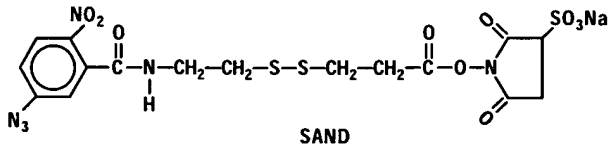
CROSS-LINKER	PRODUCT #	M.W.	SPACER ARM LENGTH	REACTIVITY	APPLICATIONS/ REFERENCES
SAED Sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamide) ethyl-1,3'-dithiopropionate	33030	621.60	23.6 Å	• NHS-ester • Phenylazide • Fluorescent	• Functionally directed region specific fluorescent labeling of proteins[189] • Assessing conformational changes in the foot protein of the sarcoplasmic reticulum by site-directed fluorescent labeling[190]
Sulfo-SAMCA Sulfosuccinimidyl 7-azido-4-methylcoumarin-3-acetate	33025	458.34	12.8 Å	• NHS-ester • Phenylazide • Fluorescent	• Specific fluorescent labeling
SAND Sulfosuccinimidyl 2-(m-azido-o-nitrobenzamido)-ethyl-1,3'-dithiopropionate	21549	570.52	18.5 Å	• NHS-ester • Phenylazide	• Demonstration of the aggregation state of Phospholipase A_2[169]



SAED



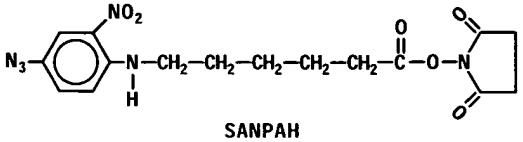
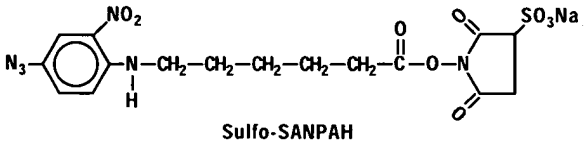
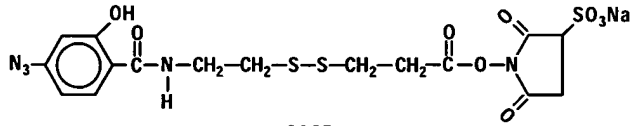
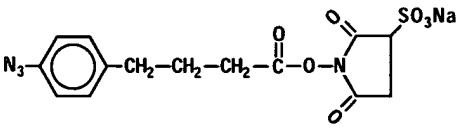
Sulfo-SAMCA



SAND

cross-linking

Table 9: Photoreactive Cross-linkers (Continued)

CROSS-LINKER	PRODUCT #	M.W.	SPACER ARM LENGTH	REACTIVITY	APPLICATIONS/ REFERENCES
SANPAH *N*-succinimidyl-6-(4'-azido-2'-nitrophenyl-amino)hexanoate  SANPAH	22588	390.95	18.2 Å	• NHS-ester • Phenylazide	• Cross-linking ligand-receptor complexes *in situ*[179] • Preparing photoactivatable glycopeptide reagents for site-specific labeling of lectins[185] • Photoaffinity labeling of the N-formyl peptide receptor binding site of intact human polymorphonuclear leukocytes[191] • Cross-linking vasoactive intestinal peptide to receptors on intact human lymphoblasts[76]
Sulfo-SANPAH Sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate  Sulfo-SANPAH	22589	492.39	18.2 Å	• NHS-ester • Phenylazide	• See applications/references for SANPAH
SASD Sulfosuccinimidyl 2-(*p*-azidosalicyl-amido)ethyl-1,3'-dithiopropionate  SASD	27716	541.51	18.9 Å	• NHS-ester • Phenylazide	• Derivatization of bacterial lipopolysaccharide[192] • Identification of the murine interleukin receptor and N-formyl peptide receptor[193] • Comparison of SASD radiolabeling techniques[194] • Cross-linking of factor V and Va to iodinated peptides[195]
Sulfo-SAPB Sulfosuccinimidyl 4-(*p*-azidophenyl)-butyrate  Sulfo-SAPB	21562	404.32	12.8 Å	• NHS-ester • Phenylazide	

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cross-linking

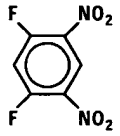
Subunit Cross-linking and Protein Structural Studies

Cross-linkers can be used to study the structure and composition of proteins in biological samples. Some proteins are difficult to study because they exist in different conformations under varying pH or salt conditions. One way to avoid conformational changes is to cross-link the subunits together. Amine-, carboxyl- or sulfhydryl-reactive reagents are employed for identification of particular amino acids or for the determination of the number, location and size of subunits in a protein. Short-to-medium spacer arm cross-linkers are selected when intramolecular cross-linking is performed. If the spacer arm is too long, intermolecular cross-linking can occur. Carbodiimides that result in no spacer arm, along with short length conjugating reagents, such as amine-reactive DFDNB (Product #21524, Table 10) or the photoactivatable amine-reactive cross-linker NHS-ASA (Product #27715), can cross-link between subunits without cross-linking to extraneous molecules if used in optimal concentrations and conditions. Slightly longer cross-linkers such as DMP (Product #20666), DMS (Product #20668), DTBP (Product #20665), DSS (Product #21555) or DSP (Product #22585) can also cross-link between subunits, but they may result in intermolecular coupling. Intermolecular cross-linking can be controlled by adjusting the amount of cross-linker and the concentration of the material to be cross-linked. Dilute protein solutions and high concentrations of cross-linker favor intramolecular cross-linking when homobifunctional cross-linkers are employed. BMH (Product #22319) and other non-cleavable, homobifunctional, sulfhydryl-reactive linkers can be used to link subunits of proteins that were joined by disulfide bonds. After reduction of the disulfides, and by cross-linking through the generated sulfhydryls, the protein will run as its full molecular mass using polyacrylamide gel electrophoresis and reducing conditions. In some circumstances, the cross-linking pattern or success may be affected by the cross-linker's solubility. Hydrophobic cross-linkers tend to cross-link more effectively in hydrophobic regions of molecules.

If the three-dimensional structure of a protein is to be determined or confirmed, cleavable cross-linkers with increasing spacer arm lengths can be used to determine the distance between two subunits. Experiments using cross-linkers with different reactive groups may indicate the locations of specific amino acids. Once conjugated, the proteins are subjected to two-dimensional electrophoresis. In the first dimension, the proteins are run under non-reducing conditions. The molecular weight of the non-reducing sample is recorded. It should be noted that some of the subunits may not be cross-linked and will run according to their individual molecular weights. Other subunits will be combined and, under nonreducing conditions, will run according to the combined molecular weight. The second dimension of the gel is then run using conditions to cleave the cross-linked subunits. The individual molecular weights of the cross-linked subunits can be determined. If the cross-linked subunits were not reduced, the pattern of the second dimension would be a diagonal. However, with the cleavable cross-linker, the cross-linked subunits will be released under reducing conditions, and the individual molecular weights of the subunits will be approximated. The cleaved subunits will be off the diagonal. The molecular weights of the individual subunits should be compared with pre-determined molecular weights of the protein subunits under reducing SDS-polyacrylamide gel electrophoresis.

cross-linking

Table 10: Bifunctional Aryl Halide

CROSS-LINKER	PRODUCT #	M.W.	SPACER ARM LENGTH	REACTIVITY	APPLICATIONS/ REFERENCES
DFDNB 1,5-Difluoro- 2,4-dinitrobenzene  DFDNB	21524	204.1	3 Å	• Aryl halide-amine and sulfhydryl-reactive	• Cross-linking phospholipids in human erythrocyte membranes[196] • Coupling peptides to albumin[197] • Studies of near neighbor relationships of proteins in the myelin membrane[198] • Cross-linking cytochrome oxidase subunits[199]

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cross-linking

Intermolecular Cross-linking for the Study of Protein Interactions and Associations

Cross-linkers are widely used for identification of near-neighbor protein relationships, ligand-receptor identification and interactions, and enzyme-substrate orientations. The cross-linkers chosen for these applications are usually longer than those used for subunit cross-linking. Homobifunctional, amine-reactive NHS-esters or imidates and heterobifunctional, amine-reactive, photoactivatable phenyl azides are the most commonly-used cross-linkers for these procedures. Occasionally, a sulfhydryl- and amine-reactive cross-linker such as Sulfo-SMCC (Product #'s 22522, 22322) may be employed if one of the two proteins or molecules is known to contain sulfhydryls. Cleavable or noncleavable cross-linkers are typically used. Because the distances between two molecules are not always known, the optimum length of the spacer arm of the cross-linker may be determined by the use of a panel of similar cross-linkers with different lengths. DSS (Product #21555) or its cleavable analog DSP (Product #22585) are among the shorter cross-linkers used for protein-protein associations. NHS-ester, phenyl azides are very useful for this type of cross-linking because they usually result in some successful, if not efficient, cross-linking. SASD (Product #27716) is a unique sulfo-NHS-ester, photoactivatable phenylazide that is iodinatable and cleavable. Its characteristics allow for detection and analysis of small quantities of protein.

Cross-linkers can be used to determine whether a particular protein is located on the surface or the integral part of the membrane. These studies are possible because water-soluble cross-linkers are membrane-impermeable, while water-insoluble cross-linkers are membrane-permeable. The experiment can be carried out by performing a conjugation reaction of a particular cell membrane preparation to a known protein or radioactive label in the presence of water-soluble or water-insoluble cross-linkers. Upon conjugation the cells may be washed, solubilized and characterized by SDS-PAGE. The gel electrophoresis results can be used to determine whether the protein of interest was conjugated. Any integral membrane protein will conjugate in the presence of a water-insoluble cross-linker, but not in the presence of water-soluble cross-linkers. Surface membrane proteins should conjugate in the presence of both water-soluble and water-insoluble cross-linkers.

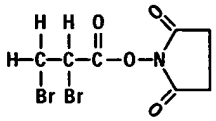
BASED (Product #21564), a homobifunctional photoactivatable phenyl azide, is one of the more versatile cross-linkers for the study of protein interactions and associations. It is cleavable and can be radiolabeled with ^{125}I using IODO-BEADS® Iodination Reagent (Product #28665). After cleavage, both of the dissociated molecules will still be iodinated. Because both reactive groups on this cross-linker are nonspecific, the cross-linking is not dependent on amino acid composition for successful conjugation.

SDBP (Product #22340) is a cross-linker that is amine-reactive at both ends, but contains two different reactive groups with varying reactivity. Please see Table 11 for more information on SDBP. The reaction is controlled by temperature. SDBP is an NHS-ester with amine reactivity that is only slightly affected by temperature; however, its second amine-reactive functional group is a dibromoacetyl group that is slow to react with amines at physiological pH at 4°C. This cross-linker can be useful for studying conformational changes in proteins.

cross-linking

Table 11: Heterobifunctional Amine-Reactive Cross-linker

CROSS-LINKER	PRODUCT #	M.W.	SPACER ARM LENGTH	REACTIVITY	APPLICATIONS/ REFERENCES
SDBP N-Hydroxysuccin-imidyl 2,3-Dibromopropionate	22340	328.96	5.0 Å	• NHS-ester • Alkyl dibromide	• Preparation of immunotoxins[200]



SDBP
M.W. 328.96
Spacer Arm 5.0 Å

References

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cross-linking

Cell Membrane Structural Studies

Cell membrane structural studies require reagents of varying hydrophobicity to determine the location and the environment within a cell's lipid bilayer. Fluorescent tags are used to locate proteins, lipids or other molecules inside and outside the membrane. Various cross-linkers with differing spacer arm lengths can be used to cross-link proteins to associated molecules within the membrane to determine the distance between molecules. Successful cross-linking with shorter cross-linkers is a strong indication that two molecules are interacting in some manner. Failure to obtain cross-linking with a panel of shorter cross-linkers, while obtaining conjugation with the use of longer reagents, generally indicates that the molecules are located in the same part of the membrane but are not interacting. Homobifunctional NHS-esters, imidates or heterobifunctional NHS-ester, photoactivatable, phenyl azides are commonly used for these procedures. Because they are membrane impermeant, sulfo-NHS-esters are not useful for cross-linking within the membrane. Imidoester cross-linkers (imidates) are water-soluble, but they are still able to penetrate membranes. DTBP (Product #20665) is an amine-reactive imidoester that is cleavable by sulfhydryls. Sulfhydryl-reactive cross-linkers may be useful for targeting molecules with cysteines to other molecules within the membrane.

EDC (Product #'s 22980, 22981), water insoluble dicyclohexylcarbodiimide, or DCC (Product #20320), and other water-soluble and water-insoluble coupling reagents are used to study membranes and cellular structure,[52,53] protein subunit structure and arrangement,[54,55] enzyme-substrate interactions,[56-58] and cell surface[59] and membrane receptors.[60,61] The hydrophilic character of EDC can result in much different cross-linking patterns in membrane and subunit studies than with hydrophobic carbodiimides such as DCC.[53,55] Often it is best to attempt cross-linking with a water-soluble and water-insoluble carbodiimide to obtain a complete picture of the spacial arrangements or protein-protein interactions involved.

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cross-linking

Immunotoxins

Specific antibodies can be covalently linked to toxic molecules and then used to target antigens on cells. Often these antibodies are specific for tumor associated antigens. Immunotoxins are brought into the cell by surface antigens and, once internalized, they proceed to kill the cell by ribosome inactivation or other means. The type of cross-linker used to make an immunotoxin can affect its ability to locate and kill the appropriate cells. For immunotoxins to be effective, the conjugate must be stable in vivo. In addition, once the immunotoxin reaches its target, it is important that the antibody be separable from the toxin to allow the toxin to kill the cell. Thiol-cleavable, disulfide-containing conjugates have been shown to be more cytotoxic to tumor cells than noncleavable conjugates of ricin A immunotoxins. Cells are able to break the disulfide bond in the cross-linker, allowing the release of the toxin within the targeted cell.

SPDP (Product #'s 21757, 21657, 21557) is a reversible NHS-ester, pyridyl disulfide cross-linker used to conjugate amine-containing molecules to sulfhydryls. For several years, this has been the "workhorse" cross-linker for production of immunotoxins. The amine-reactive NHS-ester is usually reacted first with the antibody. In general, toxins do not contain surface sulfhydryls; therefore, sulfhydryls must be introduced onto them by reduction of disulfides, which is common for procedures involving ricin A chain and abrin A chain, or through chemical modification reagents. A second SPDP molecule can be used for this purpose. It is reacted with amines on the immunotoxin, then reduced to yield sulfhydryls. Another chemical modification reagent that is commonly used for production of immunotoxins is 2-iminothiolane, also known as Traut's Reagent (Product #26101). Traut's Reagent reacts with amines and yields a sulfhydryl when its ring structure opens during the reaction.

Other water-soluble SPDP analogs, such as Sulfo-LC-SPDP (Product #'s 21650, 21649), are available for immunotoxin production, allowing for ease of use or avoidance of organic solvents. In addition, Sulfo-LC-SPDP and LC-SPDP (Product #'s 21651, 21652) have longer spacer arms and can offer better conjugation efficiency.

SMPT (Product #21558) is a reversible, NHS-ester, pyridyl disulfide cross-linker developed to provide increased stability of immunotoxins *in vivo*. The disulfide bond in SMPT is protected, making it less likely to be cleaved *in vivo* prior to reaching the antigenic target. In addition, the NHS-ester of SMPT is much more stable in aqueous solution than typical NHS-ester compounds, showing little degradation even after several hours in aqueous solution. A water-soluble long chain version of SMPT is also offered—Sulfo-LC-SMPT (Product #'s 21569, 21568).

cross-linking

Carrier Protein Hapten/ Peptide/Polypeptide Conjugates for Use as Immunogens

Pierce offers many products in this area of immunological research. Easy-to-use kits are available for coupling ligands using several different chemistries. These kits and the use of immunogens are discussed in the Antibody Production Technical Section of this catalog. There are many cross-linkers used for the production of these conjugates, and the best choice is dependent on the reactive groups present on the hapten and the ability of the hapten-carrier conjugate to function successfully as an immunogen after its injection. Carbodiimides are good choices for producing peptide carrier conjugates because both proteins and peptides usually contain several carboxyls and primary amines. Carbodiimides such as EDC (Product #'s 22980, 22981) react with carboxyls first to yield highly reactive unstable intermediates. The intermediates can then couple to primary amines. Many different carboxyl- or amine-containing small molecules can be attached to carrier proteins using this easy-to-use chemistry.

Other heterobifunctional cross-linkers can also be used to make immunogen conjugates. Often peptides are synthesized with terminal cysteines to allow for their attachment to supports or to carrier proteins through a part of the molecule that is not important for activity or recognition. Sulfhydryl-reactive, heterobifunctional cross-linkers can be coupled to carrier proteins through their other functional group and then can be linked to peptides through terminal cysteines. This method can be very efficient and yield an immunogen that is capable of eliciting a good response upon injection. A good choice of cross-linker with these characteristics is Pierce's Sulfo-SMCC (Product #22322). This cross-linker is an amine-reactive NHS-ester that contains a cyclohexyl group in its spacer and a very stable maleimide group at the other end of the molecule. The maleimide of Sulfo-SMCC is more stable than the maleimide on other NHS-ester maleimide cross-linkers because of the stability imparted by the cyclohexyl ring. Pierce uses Sulfo-SMCC to produce its entire selection of Maleimide Activated Carrier Proteins and Kits. Please see the Antibody Production Technical Section for additional information. Other cross-linkers that can be used to make immunogens are MBS (Product #'s 22510, 22310), SMPB (Product #'s 22316, 22315) and GMBS (Product #22314). Water-soluble analogs are also available, including Sulfo-MBS (Product #'s 22313, 22312), Sulfo-SMPB (Product #'s 22318, 22317) and Sulfo-GMBS (Product #22324).

SDBP (Product #22340) is a cross-linker that is amine-reactive at both ends, but contains two different reactive groups with varying reactivity. The reaction is controlled by temperature. SDBP is an NHS-ester with amine reactivity that is only slightly affected by temperature; however, its second amine-reactive functional group is a dibromoacetyl group, which is slow to react with amines at physiological pH at 4°C. A possible application for this cross-linker is to allow the NHS group to react with amines on the carrier protein. After quick removal of the excess cross-linker from the carrier protein, an amine-containing hapten can be added to the solution, and the reaction can be allowed to warm to room temperature, then proceed for several hours.

cross-linking

Solid-Phase Immobilization

Proteins, peptides and other molecules can be immobilized on solid-phase matrices for use as affinity supports or for sample analysis. The matrices may be agarose, beaded polymers, polystyrene plates or balls, porous glass or glass slides, and nitrocellulose or other membrane materials. Some supports can be activated for direct coupling to a ligand. Other supports are made with nucleophiles or other functional groups that can be linked to proteins or other ligands using cross-linkers. Carbodiimides such as DCC (Product #20320) and EDC (Product #'s 22980, 22981) are very useful for coupling proteins to carboxy- and amine-activated glass, plastic and agarose supports. Carbodiimide procedures are usually one-step methods; however, two-step methods are possible if reactions are performed in organic solvents, or if NHS (Product #24500) or Sulfo-NHS (Product #24510) are used to enhance the reaction.

EDC is useful for coupling ligands to solid supports.[62-66] It can also be used to attach leashes onto affinity supports and for subsequent coupling of ligands. Useful spacers are diaminodipropylamine (DADPA),[62] ethylenediamine, hexanediamine,[63,64] 6-amino-caproic acid,[62,65] and any of several amino acids or peptides.[62] Useful solid supports for immobilization are agarose,[62-65] plastic,[62] or cellulose matrices.[66] Leashes become necessary to overcome steric effects when the ligand is immobilized too near the matrix to allow access by the molecule to be bound. Steric effects are usually most pronounced when the ligand is a small molecule. Reaction times are generally in the range of 1-3 hours for EDC coupling of molecules to solid supports. The amide bond formed by EDC coupling is relatively stable, especially at neutral pH.

Heterobifunctional cross-linkers that can be reacted in two-steps are often more useful and efficient for producing solid-phase supports than homobifunctional cross-linkers. Amine-activated supports can be converted to sulfhydryl-reactive supports using NHS-ester maleimide cross-linkers such as Sulfo-SMCC (Product #'s 22522, 22322). For some compounds that are difficult to immobilize, it may be possible to use NHS-ester, photoactivatable, phenyl azides to attach them to amine-activated supports. The photoactivatable, phenyl azide is unreactive in the dark but, once exposed to the appropriate wavelength range of light, it becomes extremely reactive and able to nonselectively couple to almost any ligand.

The cross-linker DMP (Product #20666) has been employed in the production of immobilized antibodies on protein A or protein G columns for use as antigen purification supports.[39] After antibody binds to the Fc-binding proteins, most or all of the antibody can be oriented so that the Fab region is available for antigen recognition. DMP is applied to the bound antibody column to link the two proteins through primary amines.

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cross-linking

Protein-Protein Conjugates

One of the most widely used applications for cross-linkers is the production of protein-protein conjugates. Biological assays require methods for detection, and one of the most common methods for quantitation of results is to conjugate an enzyme, fluorophore or other molecule to a protein that has affinity for one of the components in the biological system being studied. Antibody-enzyme conjugates (primary or secondary antibodies) are among the most common protein-protein conjugates used. Secondary antibodies are relatively inexpensive and are available from Pierce (see the Antibody Ordering Section of this catalog). However, enzyme labeled primary antibodies are usually expensive and can be difficult to obtain. Many researchers find it necessary to label their primary antibodies.

There are many reagents used for the production of antibody-enzyme conjugates. These have been produced by glutaraldehyde cross-linking in one- and two-step procedures. These conjugates are easy to make but often yield conjugates that give high background in immunoassays. Carbohydrate moieties of antibodies can be oxidized and then coupled to primary amines on enzymes, such as horseradish peroxidase, in a procedure called reductive alkylation or amination. These conjugates give less background in enzyme immunoassays and are relatively easy to prepare. Some self-conjugation of antibody may occur in the protocol. Homobifunctional NHS-ester or imidoester cross-linkers can be substituted for glutaraldehyde in a one-step protocol; however, polymerization and self-conjugation are still likely to occur. Homobifunctional sulfhydryl-reactive cross-linkers such as BMH (Product #22319) and DPDPB (Product #21701) may be useful if both proteins to be conjugated contain sulfhydryls.

Heterobifunctional cross-linkers are perhaps the best choices for antibody-enzyme or other protein-to-protein cross-linking. Unwanted self-conjugation inherent when using homobifunctional NHS-ester reagents or glutaraldehyde can be avoided when using a reagent such as SMCC (Product #'s 22321, 22320) or Sulfo-SMCC (Product #'s 22522, 22322). Sulfo-SMCC is conjugated to one protein, and the second is thiolated with SATA (Product #26102) or Traut's Reagent (Product #26101). Alternatively, disulfides in the protein are reduced, and the two activated proteins are incubated together to form conjugates that are free of dimers of either protein. Any of the other NHS-ester maleimide or pyridyl disulfide cross-linkers can be substituted for Sulfo-SMCC in this reaction scheme. Heterobifunctional photoactivatable phenylazide cross-linkers are seldom used for making protein-protein conjugates because conjugation efficiencies

cross-linking

DNA/RNA Cross-linking to Proteins

Cross-linking of DNA or RNA to proteins is more limited because the reactivities of most cross-linkers favor protein-protein cross-linking over protein-DNA cross-linking. To assist in these cross-linking methods, DNA probes are often synthesized with primary amines or thiols attached to specific bases. After insertion of the bases into DNA, amine- or sulfhydryl-reactive cross-linkers can be used for their conjugation to proteins. EDC (Product #'s 22980, 22981) has been reportedly used to cross-link RNA to ribosomal protein subunits. Other specialized chemistries are reviewed in Wong's book, *Chemistry of Protein Conjugation and Cross-linking* (Product #15010).

cross-linking

Other Applications

There are many additional applications for cross-linkers that are either antiquated methods, new technologies or for more specialized needs. Older methods for peptide synthesis involve use of carbodiimide cross-linkers such as DCC (Product #20320) and EDC (Product #'s 22980, 22981) for the step-wise addition of individual amino acids to support bound peptides. Cross-linkers such as glutaraldehyde and dimethylpimelimidate have been used for tissue fixation. Newer cross-linkers are being developed that have more than two functional groups. Some trifunctionals are already reported in the literature.